tonium. Nor can the effect be through osteoblasts, which are readily damaged by other agents without concomitant effects on calcium removal(22).

Summary. A study of the distribution of plutonium-239 in bone, and its effects on bone cells has been presented and correlated with endogenous parathyroid activity. There was no measurable effect on the function of existing osteoblasts or osteoclasts during this short term (5 day) experiment. Also, incorporation of plutonium into osteoclasts did not disrupt the ability of parathyroid hormone to maintain normal calcium levels. However, it could be demonstrated that plutonium, for the first 48 hours after administration, affected certain of the undifferentiated bone cells, which in turn prevented the increased osteoclast production normally seen following endogenous parathyroid stimulation.

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Some Endogenous Parathyroid Hormone Effects Manifested by Bone *in vitro*.* (30104)

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The incubation and organ culture of bone fragments have been used by several workers to examine the behavior of bone (*in vitro*) under the influence of parathyroid extract or exogenous parathyroid hormone (1-6). However, little work has been done to assess possible endogenous parathyroid effects in such systems(5).

Recent studies from our laboratory have dealt primarily with the effects of endogenous parathyroid hormone on bone metabolism *in vivo*(8). Some studies on incubated bone tissue have also been reported(7,9,10). The purpose of the studies presented here was

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2-fold: 1) to establish a reliable, suitable system of bone incubation; and 2) to determine whether or not the influence of endogenous parathyroid hormone could be observed in bone taken from rats and incubated *in vitro*. The primary criterion used to ascertain changes in endogenous parathyroid secretion was a drop in serum and medium calcium after parathyroidectomy(11).

Materials and methods. A. *Procedures.* Adult male Holtzman rats, 150-200 g, were used in all studies. These animals were generally maintained on a normal (stock) diet†; however, in a few studies the rats were fed a calcium free diet for 3 days before the experiments.

Experimental animals were surgically parathyroidectomized using fine jeweler's forceps. Control (parathyroid intact) animals were occasionally sham operated. In one experiment parathyroid extract (Lilly's PTE) was administered subcutaneously to parathyroid intact rats in 3 doses of 100 IU each (1 hour apart); these animals were killed 13 hours after the last injection. For each experiment, control animals were always done concomitantly with the experimental animals.

In some experiments, Ca^{45} (in $CaCl_2$, 1282 mc/g) was administered subcutaneously. The rats were either injected with 10 μc of the isotope 24 hours before sacrifice, or with 40 μc 12-14 days before use.

All rats were sacrificed after blood was obtained by cardiac puncture. Femora were immediately removed and prepared for incubation in one of the following ways:

a) Preparation was similar to that outlined by Borle, Nichols, and Nichols(12); femora, thoroughly cleaned of connective tissue and muscle, were split into metaphyseal (trabecular bone) and diaphyseal (compact bone) segments and placed in separate incubation flasks containing 1.5 ml media each. Marrow was removed before incubation by washing the bone with a stream of isotonic saline.

b) Whole femora were cleaned; no attempt was made to remove the marrow. They were crudely minced with a large pair of shears and placed in flasks containing 5 ml media for incubation.

c) Femora were prepared as in either a) or b) and then frozen and thawed at least 3 times to kill the bone cells before incubation(16).

Both femoral samples from each animal were always pooled in a single incubation flask. Total preparation time for a single animal did not exceed 8 minutes. All samples were incubated in pooled rat serum (pH 7.4) at 37°C with constant shaking. The atmosphere used was 95% oxygen and 5% CO_2. Incubations were generally performed for 2-6 hours. In some experiments involving incubation of femora for 4-6 hours, aliquots of media were removed for analyses at the 2nd and 4th hours. Measurement of pH during the course of the incubations revealed that the pH was maintained at approximately 7.4.

While the serum media used possessed physiological calcium and phosphate concentrations, in a few experiments the concentrations of these 2 ions were purposely lowered. This was done by shaking the media with tertiary calcium phosphate, centrifuging the mixture at high speed, and drawing off the supernatant for serum media(7,9).

B. *Analyses.* All samples were analyzed fluorometrically for total calcium (Ca) on the Autoanalyzer‡(13). Many of the samples were also analyzed for phosphorus (P) and magnesium (Mg). Phosphorus was calculated from the total inorganic phosphate concentration which was determined by the method of Allport and Keyser(14). Magnesium was determined fluorometrically on the Autoanalyzer(15).

Ca^{45} was counted on a Nuclear-Chicago thin-window, gas flow counter (background approximately 2 counts/min). All samples were either corrected for self absorption or counted at infinite thickness.

Results. Serum calcium was determined in order to evaluate the severity of endogenous parathyroid hormone deprivation after parathyroidectomy or the extent of increased hormonal activity produced by injection of extract (Table I). Parathyroidectomy pro-

† This diet was obtained from Holtzman Co., Madison, Wisc.

‡ Technicon, Inc., New York.

TABLE I. Serum Taken from Animals When Femora Were Removed for Incubation.*

Group	No. animals	Ca (mg/100 ml)
Intact	13	9.9 ± .08
PTX†	13	7.6 ± .17
PTE‡	5	11.0 ± .17

* All values expressed as mean ± standard error ($SE = \pm\sqrt{\frac{\Sigma d^2}{n(n-1)}}$).

† PTX = parathyroidectomized 8-12 hr.

‡ PTE = parathyroid extract (Lilly's), total dose = 300 IU.

duced the expected drop in serum calcium; conversely, PTE caused a significant elevation of the serum calcium. While a continuous drop in serum calcium was observed during the 12 hours after parathyroidectomy, significant depression of the serum calcium was always detectable 4 hours after removal of the glands.

In our initial experiments, incubations were done using Krebs-Ringer bicarbonate buffer; synthetic medium has been used extensively by other workers(1-5,16). However, serum medium provided more consistent, reliable results than did the Krebs-Ringer medium. Further, we experienced the usual difficulty in preparing the latter with near-physiological (8-10 mg/100 ml) calcium concentrations. Although other workers have found a calcium-free or low calcium, buffered medium preferable, for our studies it was particularly advantageous to use 1) a medium from which the most consistent calcium values could be obtained upon analysis; and 2) a medium in which chemical dissolution of hydroxyapatite crystal was not a problem. Pooled rat serum met both of these requirements.

TABLE II. Calcium Levels in Medium After 4 Hours Incubation of Femoral Bone from Intact and Parathyroid Extract-Treated Rats.*

Group	No. animals	Ca (mg/100 ml)
Intact-methaphysis	4	6.4 ± .10
PTE-methaphysis†	5	7.3 ± .09
Intact-diaphysis	4	5.1 ± .15
PTE-diaphysis	5	5.7 ± .09
Serum pool (initial level in medium)		8.9

* All values expressed as mean ± SE.

† PTE = parathyroid extract (Lilly's), total dose = 300 IU.

A. *Metaphyseal and diaphyseal incubations.* Fig. 1 illustrates that regardless of whether the initial calcium level in the medium was extremely low (essentially Ca-free) or normal, equilibration was approached after 4 hours of incubation. The calcium maintained in the medium by metaphyseal bone was consistently higher than the level maintained by diaphyseal bone. This was true whether the bone was taken from parathyroid intact or from experimental animals (Tables II, III).

TABLE III. Calcium Levels in Medium After 4 Hours Incubation of Femoral Bone from Intact and Parathyroidectomized Rats.*

Group	No. animals	Ca (mg/100 ml)
A. Live bone		
Intact-metaphysis	13	6.3 ± .13‡
PTX†-metaphysis	13	6.0 ± .11‡
Intact-diaphysis	12	5.1 ± .13§
PTX-diaphysis	13	4.7 ± .08§
B. Dead bone		
Frozen-metaphysis	4	5.6 ± .20
Frozen-diaphysis	4	4.8 ± .17
Serum pool		7.6

* All values expressed as mean ± SE.

† PTX = parathyroidectomized 5-10 hr.

‡ Differ from each other by $p < .100 > .050$.

§ Differ from each other by $p < .025 > .010$.

In one experiment the effect of a pharmacological dose of PTE was tested (Table II), primarily to compare this technique of bone incubation to those used by other workers (1-5,9,16). Administration of PTE to rats 13 hours before femoral incubation caused an elevation in the media calcium maintained by both metaphyseal and diaphyseal bone. A similar effect has also been observed by others for preparations incubated in synthetic media(2,5).

B. *Effect of parathyroidectomy.* Metaphyseal and diaphyseal bone from intact rats tended to maintain higher calcium levels in the medium than corresponding samples from parathyroidectomized animals (Table IIIA). However, the difference between the intact and parathyroidectomized groups was more significant when diaphyseal bone was incubated (Students 't' test, $p<0.025>0.010$) than when metaphyseal bone was incubated ($p<0.100>0.050$). The consistent mainte-

nance of higher calcium concentrations by metaphyseal bone compared to diaphyseal bone was evident even when dead bone was incubated (Table IIIB).

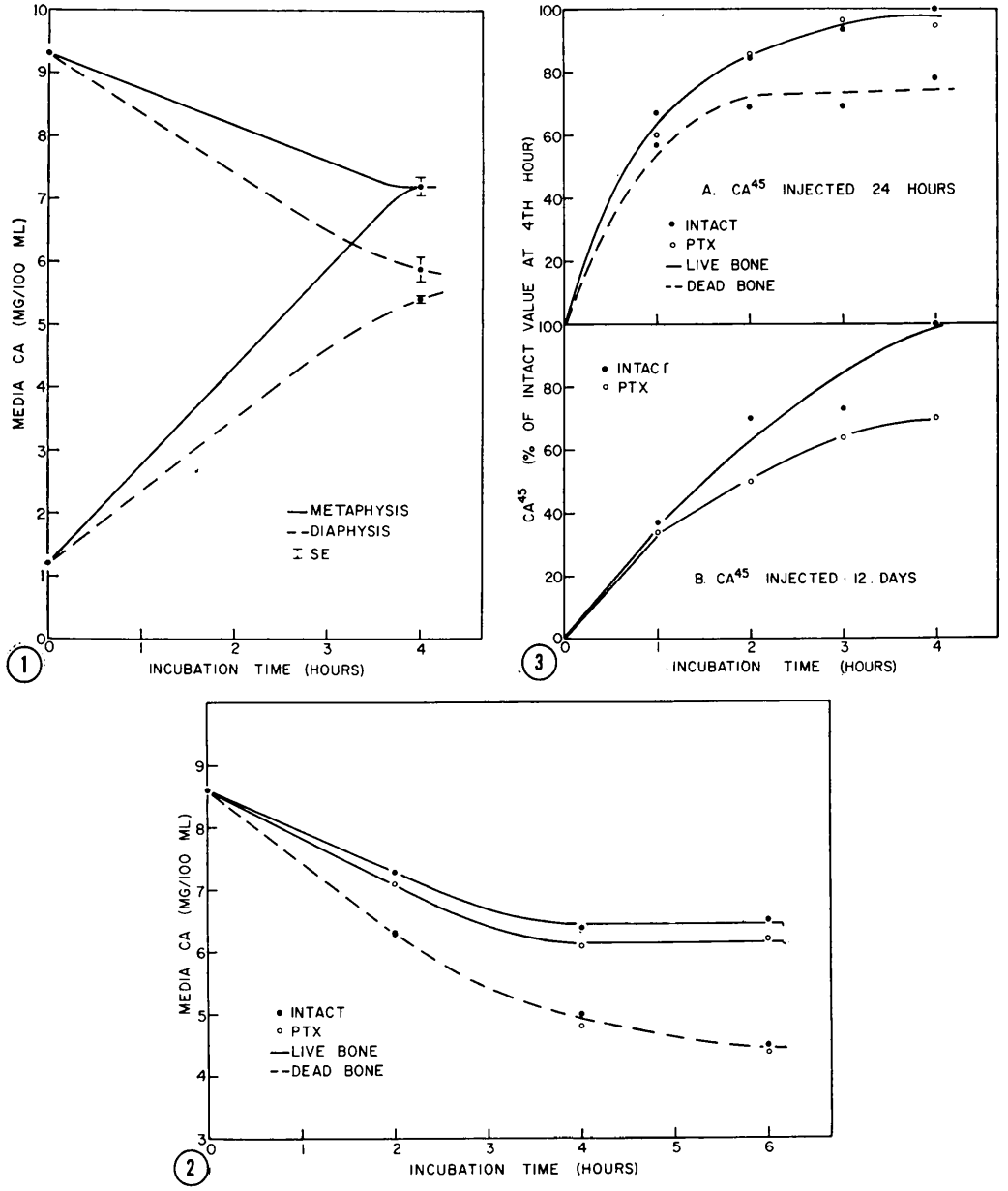

FIG. 1. Incubation of metaphyseal *vs* diaphyseal bone in media with normal and low initial calcium levels. SE = Standard error of mean ($\pm \sqrt{[\Sigma d^2/n(n-1)]}$). Metaphysis differs from diaphysis by $p < 0.005$ at hour 4 (Students' 't' test).

FIG. 2. Calcium levels in medium during incubation of whole femur fragments. PTX = parathyroidectomized; Dead Bone = frozen-killed bone. Intact and PTX live bone differ by $p < 0.005$ at hours 2, 4 and 6. Dead bone differs from live bone (PTX) by $p < 0.001$ by hour 2.

FIG. 3. Ca^{45} transfer from bone to medium during incubation of whole femur fragments. PTX = parathyroidectomized; Dead Bone = frozen-killed bone. A. Ca^{45} injected 24 hr before sacrifice of animals. Dead bone differs from live bone by $p < 0.025$ at hour 2, $p < 0.005$ at hours 3 and 4. B. Ca^{45} injected 12 days before sacrifice of animals. PTX differs from intact by $p < 0.050$ at hour 2, $p < 0.010$ at hour 3, $p < 0.005$ at hour 4.

TABLE IV. Radioactivity Found in Medium After Incubation of Femoral Bone from Intact and Parathyroidectomized Rats Injected with Ca^{45} for 14 Days.

Group	No. animals	Incubation time (hr) 2	3	4
		(%)	(%)	(%)
Intact-metaphysis	4	94.4 ± 6.6*	98.5 ± 6.6	100 ± 10.7
PTX-metaphysis†	4	81.6 ± 3.4	94.9 ± 9.8	102 ± 5.3
Intact-diaphysis	4	58.6 ± 4.9	65.4 ± 5.8	75.2 ± 4.1‡
PTX-diaphysis	4	51.9 ± 2.3	59.8 ± 3.8	64.3 ± 2.1‡

* For ease of comparison, all values are expressed as % of intact-metaphysis value at 4th hour of incubation (mean ± SE).
† PTX = parathyroidectomized (4 hr).
‡ Differ from each other by p = .025.

In one series of experiments, femora were removed from animals that had been injected with Ca^{45} 2 weeks before sacrifice, divided into metaphyseal and diaphyseal portions, and incubated (Table IV). In all groups the amount of Ca^{45} transferred from bone to the medium increased during the incubation. Metaphyseal bone released significantly more radioactivity into the medium than did diaphyseal bone. This might not be totally unexpected, since injected Ca^{45} is initially incorporated primarily in the metaphysis(7,17, 18). However, the only detectable effect of parathyroidectomy was after 4 hours of incubation, where the intact diaphyseal bone had released more Ca^{45} into the medium than the diaphyseal bone from parathyroidectomized rats ($p=0.025$).

C. *Whole femora incubations.* Fig. 2 and 3 illustrate results obtained from experiments similar to those already presented. Femora taken from parathyroid intact animals were able to maintain a significantly higher calcium in the medium over 6 hours of incubation than were those taken from parathyroidectomized rats ($p<0.005$, Fig. 2, Live Bone). Frozen-killed femoral fragments not only showed no effects of parathyroidectomy, but also equilibrated at a lower level than did the live bone. This indicated 1) that when live bone was incubated, media calcium was partially maintained by cellular mediation (both intact and parathyroidectomized); and 2) endogenous parathyroid hormone (Live Bone, parathyroid intact group) influenced only cellular mediated processes rather than the inherent, physical solubility of the hydroxyapatite crystal (Dead Bone). Similar results have been reported previously (5, 16).

When rats were injected with Ca^{45} 24 hours before their femora were removed and incubated, the amount of radioactivity transferred to the medium was the same for both parathyroidectomized and parathyroid intact groups (Fig. 3A). However, dead bone contributed significantly less Ca^{45} to the serum medium. If the Ca^{45} was administered 12 days before femoral incubation, the amount of radioactivity transferred to the medium was significantly higher for the intact group compared to the parathyroidectomized group (Fig. 3B). These results are similar to those obtained from *in vivo* studies reported previously from this laboratory(7,18). Apparently "parathyroid hormone acts in bone at the level of previously formed bone crystal" (18); therefore, parathyroidectomy effects on Ca^{45} removal from bone are apparent only after the isotope has been incorporated for longer than 24 hours.

D. *Effects of parathyroidectomy on P and Mg concentrations.* Phosphorus levels in the serum of rats were elevated after parathyroidectomy (Table 5A). Phosphorus concentrations in the incubation medium were the same whether metaphyseal or diaphyseal bone was incubated (Table 5B); however, femora from parathyroidectomized rats maintained a consistently higher medium P than did femora from parathyroid intact rats. Although other workers have noted similar effects on *in vitro* phosphorus concentrations(2,5), increased *serum* phosphorus after parathyroidectomy is known to be due to the decreased renal excretion of phosphate(19). We are,

TABLE V. Phosphorus and Magnesium Levels in Animal Serum and in Medium (after incubation of femoral bone for 4 hr).

Group	P (mg/100 ml)	Mg (meq/l)
A. Animal serum		
Intact	10.0 ± .14 (13)*	1.61 ± .03 (10)*
PTX†	11.9 ± .22 (13)	1.38 ± .05 (8)
B. Incubation medium		
Intact metaphysis	8.9 ± .10 (13)	1.71 ± .01 (9)
PTX-metaphysis	9.4 ± .10 (13)	1.74 ± .03 (9)
Intact diaphysis	8.8 ± .09 (12)	1.58 ± .02 (9)
PTX-diaphysis	9.3 ± .08 (13)	1.59 ± .02 (8)
Serum pool	9.8	

* No. in brackets = No. of animals.
† PTX = parathyroidectomized 5-10 hr.

therefore, unable to explain why femora from parathyroidectomized animals maintain higher phosphorus levels *in vitro*.

Serum magnesium changes mimicked calcium changes after parathyroidectomy (Table 5A), indicating that the serum level of this ion is determined to some extent by the equilibration of the bone crystal with the medium. Magnesium is one of several cations known to substitute for calcium in the hydration layer of the hydroxyapatite crystal complex(20). Magnesium levels in the incubation medium resembled calcium levels, in that metaphyseal and diaphyseal bone were significantly different (Table 5B). No detectable differences in Mg between the media of intact and parathyroidectomized groups were observed.

Discussion. These studies offer a system of bone incubation which, in many ways, reflects the *in vivo* condition. The use of pooled serum, rather than synthetic medium, proved extremely advantageous, enabling a reliable evaluation of parathyroid effects by analysis of calcium changes in the serum medium. Experiments involving parathyroidectomy, pretreatment of rats with PTE, and a comparison of live and dead bone indicate that this particular method of bone incubation is, in some ways, comparable to the methods used by other workers(1-7,9,16). That dead bone equilibrates at a lower calcium level than does live bone has been demonstrated previously by Bassett and Nordin(16), Wimer(9) and Raisz *et al*(5). Raisz *et al*(5) have also demonstrated that membranous bone from normal, parathyroidectomized and PTE-treated rats exhibit different calcium and phosphorus levels *in vitro*.

From the studies presented here, several new observations can be made concerning parathyroid-bone relationships. The first concerns the difference between metaphyseal and diaphyseal bone. The calcium equilibration studies illustrate graphically that these two regions of bone behave quite differently *in vitro*. Metaphyseal bone equilibrates at a level consistently higher than does diaphyseal bone. Since this occurs with dead bone as well as with live bone, cellular activity is not wholly responsible for the difference. Further, the two areas equilibrate at different levels irrespective of the initial medium calcium concentration; thus their well known differential surface areas cannot be held accountable for this phenomenon, since this would influence the rate at which equilibrium is attained but not the final equilibration level. Therefore, the observed differences must be ascribed to a difference in solubility between these 2 regions of femoral bone. It is particularly interesting to note that this "apparent" solubility is highest in trabecular (metaphyseal) areas of the femur, considering that this is also the region where apatite nucleation and accretion proceed most rapidly.

Secondly, bone taken from parathyroid intact rats is able to maintain higher calcium concentrations than bone obtained from parathyroidectomized animals (for at least 6 hours). Since the effects of parathyroidectomy can be detected whether the bones are removed 4 or 12 hours after the operation, the biological half life of endogenous parathyroid hormone appears to be relatively short. It is our contention that a drop in serum- or medium-calcium and Ca^{45} concentration within a few hours after parathyroidectomy indicates continuous, prolific endogenous parathyroid hormone secretion in parathyroid intact rats(11). It should be noted that such a viewpoint concerning continuous endogenous hormonal secretion is not su-

ported by all workers(21). However, if this contention is valid, then: 1) endogenous parathyroid hormone is still present in the bone incubated from parathyroid intact rats; 2) the target organ (bone) effect is still present in this bone—although the hormone molecule itself may not be; or 3) a combination of 1) and 2) may exist.

Previous reports from this and other laboratories have emphasized maintenance of a "basic equilibration" level between plasma and bone by the parathyroidectomized animal(22,23). This is confirmed by these and previous studies(5) which demonstrate that bone taken from parathyroidectomized animals maintains media calcium at a much higher level than does bone devoid of live cells. Therefore, it must be concluded that the maintenance of this basic equilibration level is mediated by cellular activity. The Ca^{45} data presented in Fig. 3 provide supportive evidence for this conclusion and indicate, further, that this basic equilibration level is partly maintained *in vitro* by active dissolution of calcium salts. Incubated dead bone contributes significantly less radioactivity to the medium than does bone from either parathyroidectomized or parathyroid intact groups. Furthermore, effects of parathyroidectomy on Ca^{45} transfer into the medium can be detected only after the isotope has been incorported over a period exceeding 24 hours. These observations concerning Ca^{45} transfer necessitate the consideration that at least 2 distinct, *active* processes of calcium removal are operable in bone: 1) a process which, at present, is *not* demonstrably influenced by endogenous parathyroid hormone (24 hour Ca^{45} studies) involving the breakdown of recently deposited calcium salts; and 2) a process which is influenced by endogenous parathyroid hormone (12 day Ca^{45} studies) involving the breakdown of preformed hydroxyapatite crystal(23).

While the data are not sufficiently conclusive to permit a definite localization of the endogenous parathyroid hormone effect on bone, the studies of parathyroid intact *vs* parathyroidectomized animals (analyses of both total calcium and Ca^{45}) indicate that though effects of endogenous parathyroid hormone can be detected in all regions of femoral bone, the effects noted on calcium *homeostasis* are much more marked when diaphyseal (compact) bone is used. This conclusion is in accord to some extent with a previous report that the morphological changes, caused by parathyroid hormone in the active, remodelling areas of (metaphyseal) bone, are quite independent of the hormonal effect on rapid calcium mobilization from bone(24).

Summary. A system of bone incubation is described in which pooled rat serum was used for the incubation of femoral fragments. The following results were observed: 1. *In vitro* calcium and Ca^{45} equilibration studies on metaphyseal and diaphyseal femoral rat bone indicate that metaphyseal bone equilibrates at a higher level than does diaphyseal bone. 2. Incubated bone taken from parathyroid intact animals maintains higher calcium levels *in vitro* than bone from parathyroidectomized rats; this is also the case for Ca^{45} injected longer than 24 hours before sacrifice and subsequent incubation. If the Ca^{45} is injected less than 24 hours before sacrifice, the same amount of radioactivity is released into the medium for both intact and parathyroidectomized groups. Dead bone, however, always releases less radioactivity than live bone. 3. These studies indicate that endogenous parathyroid hormonal effects are detectable when bone is removed from parathyroid intact rats and incubated. These effects are exerted through an influence on cellular mediated processes; however, bone from parathyroidectomized rats also maintains media calcium levels partly through energy requiring processes. 4. Evidence presented illustrates 2 distinct processes of bone salt removal exist, one of which is influenced by endogenous parathyroid hormone. Data also imply that diaphyseal (compact) bone may be the primary site of parathyroid hormone influences on calcium homeostasis.

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Influence of Endogenous Parathyroid Hormone on Citrate and Lactate Production by Bone *in vitro*.* (30105)

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The specific mechanism by which the parathyroid glands influence calcium homeostasis has not been established. Discovery of calcium-lowering factors from the parathyroids, thyroids, and adrenals(1,2,3) have made the problem even more complex. Clearly, however, the function of the classical parathyroid hormone is to maintain the circulating calcium level by altering kidney thresholds and mobilizing bone calcium. Suggestions for explaining the bone salt mobilization have included mitochondrial transport(4), alterations in RNA(5), and changes in bone cell carbohydrate metabolism(6,7,8). Various workers have reported apparently conflicting data on the effects of parathyroid extract on glucose breakdown by incubated bone tissue. Some have found that citrate production by the cells was altered(9), others have reported changes in lactate production(7,8), and still others have found no effect(10). In contrast to the above studies which were done primarily with the administration of parathyroid extract, the work reported here was carried out to determine whether or not changes in the level of *endogenous* parathyroid hormone secretion would have any effect on the production of citrate and lactate by bone tissue incubated *in vitro*.

Materials and methods. Male Holtzman rats, 180-200 g, were used throughout these studies. To alter production of endogenous parathyroid hormone, the animals were subjected to one or more of the following treatments: parathyroidectomy, nephrectomy, and/or peritoneal lavage. All surgery was performed under light ether anesthesia. Nephrectomy was done through a single, mid-ventral incision; parathyroidectomy was

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